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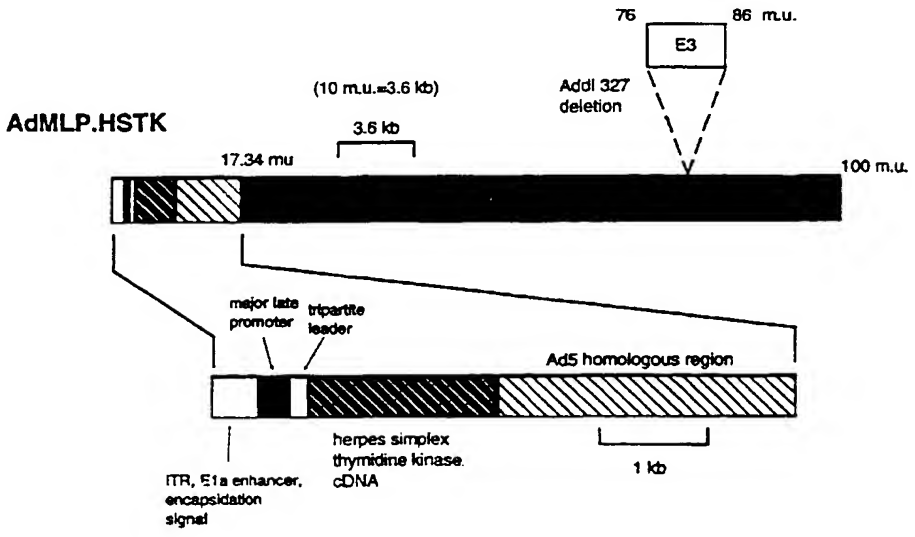

(57) Abstract

The present invention provides a method of selectively expressing DNA in neointimal cells in an injured blood vessel of a subject comprising administering a replication-deficient recombinant adenovirus which functionally encodes the DNA to the blood vessel at the site of injury, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect neointimal cells and thereby selectively express the DNA in neointimal cells. In particular, the invention provides administering a replication-deficient recombinant adenovirus which functionally encodes a DNA encoding a protein or an antisense ribonucleic acid. This method can be used to treat restenosis and, relatedly, to prevent neointimal cell proliferation.

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SELECTIVE ADENOVIRAL MEDIATED GENE TRANSFER INTO VASCULAR NEOINTIMA

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to a method of delivering DNA to neointimal cells in injured blood vessels. In particular is provided a method of decreasing or inhibiting the proliferation of neointimal cells by delivering DNA that causes the decrease or inhibition of proliferation of neointimal cells, using replication-deficient recombinant adenoviral vectors, thus treating restenosis.

BACKGROUND ART

Restenosis, a process characterized by smooth muscle cell (SMC) proliferation associated with arterial injury, remains a major obstacle to the long-term success of coronary angioplasty. The injury activates medial SMCs, which begin to migrate and proliferate to form a neointima. Angioplasty failure rates of 25% to 50% within six months have been reported and confirmed by several authors (1-5). In addition, restenosis is largely responsible for the treatment failures of distal peripheral arterial reconstructions, and of newer techniques which include atherectomy, stent implantation, and laser angioplasty.

In uninjured vessels, smooth muscle cells (SMC) are normally located in the medial layer where they infrequently divide. Histological examination of vascular lesions from both animal models and humans indicates that following injury, SMCs are induced to migrate and proliferate. Over the next several weeks, these SMCs form a new layer of cells, termed the "neointima". It is thought that in patients with restenosis it is largely the expansion of this neointimal layer, consisting of SMC, extracellular matrix, and recruited inflammatory cells, that eventually results in the reduction of blood flow and recurrence of ischemic symptoms.

Previous attempts to modulate this cellular proliferation have included various mechanical and pharmacologic therapies, which have been the subject of several reviews (6-9). More recently, many efforts have been directed against various growth factors, their receptors, or cellular proto-oncogenes thought to play an important role in SMC proliferation (10-19). Although several of these methods have shown encouraging *in vitro*, and more recently, *in vivo* results, all approaches have both practical and theoretical drawbacks. Hence, while one or more of these therapeutic strategies may ultimately show clinical efficacy, the need for more powerful and specific approaches is compelling.

Gene therapeutic techniques offer the promise of efficiently transferring genes, whose products may convey therapeutic benefit, to specific groups of cells. Previous efforts to directly transduce arterial segments *in vivo* have used liposomal or retroviral methods to transfer marker genes into endothelial or SMCs (20-24). The feasibility of such efforts, however, has been limited by a low transfection efficiency. In *in vivo* models, estimates of gene transfer into arterial segments range from fewer than 1 in 10,000 cells transduced with retroviral methods (24) to fewer than 1 in 1,000 cells using liposomes.

Replication deficient recombinant adenoviral vectors have previously been shown to be efficient for transferring exogenous genes to a wide variety of cells *in vivo* (25-36). Such vectors can be manipulated so as to encode for recombinant gene products up to 7.5 kilobases (kb) in length (37). The recombinant virus can be propagated in certain mammalian cell lines that serve to complement the growth of replication effective adenovirus. Additionally, transduction by adenovirus, as opposed to retrovirus, does not depend on active replication of the host cell (37,38).

The present invention provides for the use of adenoviral vectors for selective and efficient expression of DNA in neointimal cells at the site of an injury. This expression can be utilized as a much needed means to treat restenosis and thus increase the success of angioplasty, atherectomy, stent implantations and the like.

SUMMARY OF THE INVENTION

The present invention provides a method of selectively expressing DNA in neointimal cells in an injured blood vessel of a subject comprising administering a replication-deficient recombinant adenovirus which functionally encodes the DNA to the blood vessel at the site of injury, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect neointimal cells and thereby selectively express the DNA in neointimal cells. In particular, the invention provides administering a replication-deficient recombinant adenovirus which functionally encodes a DNA encoding a protein or an antisense ribonucleic acid.

The instant invention also provides a method of treating restenosis in an injured blood vessel of a subject comprising administering to the blood vessel a replication-deficient recombinant adenovirus which functionally encodes a DNA which can decrease the proliferation of neointimal cells, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect and express the DNA in neointimal cells, thereby decreasing or inhibiting the proliferation of neointimal cells and treating restenosis.

Additionally, the present invention provides a method of preventing or reducing neointimal cell proliferation in an injured blood vessel of a subject comprising administering to the blood vessel a replication-deficient recombinant adenovirus which functionally encodes a DNA which can decrease the proliferation of neointimal cells, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect and express the DNA in neointimal cells, thereby preventing the proliferation of neointimal cells. Also provided is the use of this method of preventing neointimal cell proliferation to treat primary atherosclerosis.

Additionally provided is a method of reducing neointimal cell proliferation in an injured blood vessel of a subject comprising administering to the blood vessel (1) a replication-deficient recombinant adenovirus which functionally

encodes herpes simplex virus thymidine kinase, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect neointimal cells, and (2) an effective amount of ganciclovir, thereby reducing the proliferation of neointimal cells that express the herpes simplex virus thymidine kinase gene, as well as of non-expressing neighboring neointimal cells.

The present invention further provides a method of screening DNA for the ability to inhibit proliferation of or to have cytotoxic effects on neointimal cells comprising administering to an injured blood vessel in a subject at the site of injury a replication-deficient adenovirus which functionally encodes the DNA, for a time sufficient for the adenovirus to selectively infect neointimal cells; and detecting inhibition of proliferation of or toxicity to the neointimal cells, such inhibition or toxicity indicating a DNA having the ability to inhibit proliferation of or to have cytotoxic effects on neointimal cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows time dependence of adenovirus-mediated gene transfer into cultured vascular SMCs. Quiescent SMCs were exposed to media containing Ad.RSVβgal for varying time intervals as described in the text and percent of cells that are nuclear dominant blue staining are plotted as a function of time viral incubation. Five hundred cells were counted in duplicate flasks that had been stained for β-gal activity. Averages from duplicate flasks varied less than 5% from the mean. Data shown are from one experiment but are representative of 3 different experiments.

Fig. 2 shows the effect of AdMLP.HSTK on vascular SMC growth *in vitro*, indicating the growth curve of AdMLP.HSTK infected cells (100 pfu/cell) treated with increasing concentrations of ganciclovir (gcv) (0, 1, and 10 μg/ml).

Fig. 3 shows the growth curve of cells infected with the control adenovirus vector, AdRSV.ßgal (100 pfu/cell) treated with increasing concentrations of ganciclovir (0, 1, and 10 µg/ml).

Fig. 4 shows the growth curve of cells infected with increasing concentrations of AdMLP.HSTK (0, 1, 10, and 100 pfy/cell) and treated with 10 µg/ml of ganciclovir.

Fig. 5 shows the average ratio of neointimal to medial areas of balloon injured rat carotid arteries, determined at 21 days after injury by digital planimetry using the section with the greatest neointimal area.. An ANOVA of the six groups was significant (p=0.027). Pair-wise Bonferroni corrected t-test between groups are shown above.

Fig. 6 shows the construction of AdMLP.HSTK, which was constructed by performing homologous recombination in 293 cells. (mcs=multiple cloning site; ITR=inverted terminal repeat; MLP=major late promoter; Ad5=adenovirus type 5).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples and Figures included therein.

The present invention provides a method of selectively expressing DNA in neointimal cells in an injured blood vessel of a subject comprising administering a replication-deficient recombinant adenovirus which functionally encodes the DNA to the blood vessel at the site of injury, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect neointimal cells and thereby selectively express the DNA in neointimal cells. Any blood vessel injured sufficiently to cause neointimal cell formation is contemplated as "an injured blood vessel" herein.

The administration of the adenovirus can be performed prior to the time of injury, concurrently with the time of injury, or after the injury to the blood vessel. A preferable administration period is from about 1 week prior to injury to about 4 weeks after injury. A more preferable administration period is from about the time of injury to about 20 days or from about the time of injury to about 15 days after the blood vessel is injured, and more preferably, from about the time of injury to about 7 days following injury. For practical considerations, it can be preferable to administer the adenovirus at the time of injury. Administration can preferably be performed during the time period in which cells within the injured artery wall are proliferating. Preferable administration times for a subject can readily be determined given the teachings herein. By administration "at the site of injury" is meant such that the adenovirus contacts the injured walls of the blood vessel, including the neointimal cells formed. Preferably, the adenovirus is administered such that the adenovirus remains at the site of injury, *i.e.*, in contact with the region of the vessel wall having neointimal cells, a sufficient time for the adenovirus to selectively infect neointimal cells. The upper limit on such contact time will typically be a factor of patient comfort, since administration can be performed for extended periods of time; therefore, a preferable amount of time for contact of adenovirus with neointimal cells is from about 0 minutes to about several hours, more preferably from about 1 minute to about 2 hours, and more preferably from about 15 minutes to about 60 minutes.

The exact method of administration can affect how the virus is placed in contact with neointimal cells. For example, one known means of administering to the bloodstream is by use of commercially available catheters for dwelling solutions. Delivery of such solutions by catheter is standard and known in the art (*see* e.g., 50). When using such catheters, the catheter is placed precisely at the site of injury within the blood vessel to allow for the adenovirus to selectively infect the neointimal cells. Other administration means, for example, as described herein, can be used. Therefore, any administration method, for any amount of time that causes the recombinant to contact neointimal cells for a time sufficient to selectively infect the neointimal cells is contemplated herein.

By "selectively" infecting neointimal cells is meant that the adenovirus infects neointimal cells while only minimally, if at all, infecting non-neointimal cells. Specific examples of such selective infection are provided herein. In particular, it is meant that primarily neointimal cells are infected. Generally, minimal, e.g., less than about 10% and usually less than about 1%, infection occurs in surrounding endothelial cells or medial SMCs and in distal organs, such as the heart, brain or liver. By "selective infection" is also meant that at least 20% neointimal cells be infected, and preferably at least 50%.

"Expressing DNA", as used herein, includes the transcription of the DNA into a ribonucleic acid if an antisense construct is used. "Expressing DNA" also includes both transcription of DNA into an mRNA and translation of the mRNA into protein when an expressed protein is desired. DNA can be genomic DNA or complementary DNA (cDNA). Therefore, by "selectively expressing DNA in neointimal cells" is meant that the protein or antisense RNA is produced selectively in the neointimal cells in the blood vessel.

A "replication-deficient recombinant adenovirus, which functionally encodes the DNA", includes any adenovirus incapable of replication, many of which, such as Ad5, are known in the art, into which has been cloned a DNA sequence to be expressed after infection of a cell by the adenovirus. By "functionally encodes" is meant that the DNA is capable, for example, of transcription and translation into the encoded protein or capable of transcription into an antisense RNA, in the infected cells. Thus, the DNA has any necessary sequences for expression (i.e., promoter, etc.).

Replication deficient recombinant adenoviruses containing the DNA of choice can be constructed by standard molecular techniques. For example, this invention demonstrates the use of a cotransfection system with the plasmid pJM17, which was first described in 1988. In this method, the gene (cDNA) of interest is first cloned into a shuttle vector, in which the cDNA of interest is flanked by adenoviral sequences. This shuttle plasmid is then cotransfected with the pJM17 plasmid into 293

cells. The pJM17 plasmid contains the entire Ad5 DNA molecule, but contains an insert in the E1 region of the virus resulting in a viral genome that exceeds the packaging constraints of adenoviral capsids. Recombination of the shuttle plasmid with pJM17 can result in a recombinant virus which can now be packaged as long as the cDNA is less than a certain size (usually ≤ 2kb). Such recombinant viruses can be detected as plaques on a lawn of 293 cells. Viral particles can subsequently be amplified so as to be produced in large amounts (49).

As noted above, a DNA encoded by an adenovirus herein can encode a protein or an antisense ribonucleic acid. DNA encoding a protein can be selected according to the protein desired for expression in neointimal cells, for example, for therapeutic purposes. A DNA encoding an antisense RNA can be selected according to a protein desirable to inhibit or decrease in neointimal cells, by providing an RNA that will selectively bind to the cellular mRNA encoding such protein. Control regions, such as enhancers and promoters, can be selected according to the cell or tissue in which it is to be expressed, as is known in the art.

Relatedly, the present invention also includes a method of treating restenosis in an injured blood vessel of a subject comprising administering to the blood vessel a replication-deficient recombinant adenovirus which functionally encodes a DNA which can decrease the proliferation of neointimal cells, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect and express the DNA in neointimal cells, thereby decreasing or inhibiting the proliferation of neointimal cells and treating restenosis. A DNA which can decrease the proliferation of neointimal cells can encode a protein which will cause a decrease in proliferation of the neointimal cells. Alternatively, an antisense RNA corresponding to a cellular protein can be provided, which will cause a decrease in the proliferation of the neointimal cells. Many examples of such proteins are known, and, for example, can include the herpes virus thymidine kinase, cytosine deaminase, the dominant-negative ras gene product, and nitric oxide synthase, all of which are known in the art. Futhermore, combination treatments of infecting cells with an coding construct encoding a gene

product that can act on an administered prodrug to render the prodrug cytotoxic, can be utilized. For example, if a construct including the herpes simplex virus thymidine kinase gene is utilized, administration of the prodrug ganciclovir, which is phosphorylated by the thymidine kinase gene product to form a nucleoside analog that in turn inhibits DNA replication, can be performed before, concurrently with or after administration of the adenovirus. For another example, the cytosine deaminase coding sequences, known in the art, can be cloned into an Ad5 vector wherein the coding sequences are under the control of a strong promoter, such as the CMV promoter, and the cytosine deaminase is expressed in infected cells. This adenovirus can be administered as described herein and the nontoxic prodrug 5-fluorocytosine (5FCyt) can also be administered. Upon expression of the cytosine deaminase, the cytosine deaminase converts the 5FCyt to 5 fluorouracil (5FUra), which can then kill the cytosine deaminase- expressing proliferating cells, as well as neighboring proliferating cells.

Examples of useful antisense RNA include constructs that target c-myc, c-myb, CDC2 and PCNA (10-18). Many other useful constructs will be apparent to one of skill in the art, based upon other uses of such antisense molecules for cytotoxicity. Binding of the antisense molecule to the target mRNA destroys the mRNA. Specific examples of antisense deoxyoligonucleotide constructs which have been successfully utilized generally for cytotoxicity are provided herein as SEQ ID NOs: 1 (against c-myc), 2 (against PCNA), and 3 (against c-myb). The human homologs of these can be particularly useful for human subjects. Antisense oligodeoxynucleotide constructs that target these example molecules, and, specifically, that span the homologous regions exemplified in the sequence listings, can be utilized. Preferable antisense-encoding constructs can encode full-length, or at least around 1000 base, complements to target sequences; however, smaller length sequences down to oligonucleotide size can be utilized.

Specifically, a DNA that, when expressed, is inhibitory or cytotoxic to the neointimal cells can be used to decrease proliferation of neointimal cells. Any DNA of choice can be readily screened for usefulness by inserting it into a herein described

adenovirus vector and administering it to cells and/or animals as herein described to monitor and quantify its antiproliferative effects. Appropriate concentrations for inhibition of proliferation can thus readily be determined, given the data herein. In alternative embodiments, a smooth muscle cell-specific promoter, for example, can be used with the coding sequence to thereby limit expression of the DNA, or a stronger (for example, the CMV promoter) or weaker promoter than exemplified herein can be used. Additionally, a recombinant vector that encodes for multiple gene products, some with antiproliferative effects and others with, *e.g.*, antithrombotic or antiplatelet effects, can be useful in treating a patient with vascular injury.

Additional treatments can be used in conjunction with the administration of the adenovirus. For example, combination treatments of infecting cells with an coding construct encoding a gene product that can act on an administered prodrug to render the prodrug cytotoxic, can be utilized. For example, if a construct including the herpes simplex virus thymidine kinase gene is utilized, administration of the prodrug ganciclovir, which is phosphorylated by the thymidine kinase gene product to form a nucleoside analog that in turn inhibits DNA replication, can be performed before, concurrently with or after administration of the adenovirus, thus inhibiting the proliferation of the cells which take up the phosphorylated analog. If a construct including the cytosine deaminase gene is utilized, administration of 5FCyt, which is converted to 5FUra by the cytosine deaminase, can be performed before, concurrently with or after administration of the adenovirus to inhibit proliferation of cells which take up the 5FUra.

The present invention further provides a related method of preventing or reducing neointimal cell proliferation in an injured blood vessel of a subject comprising administering to the blood vessel a replication-deficient recombinant adenovirus which functionally encodes a DNA which can decrease the proliferation of neointimal cells, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect and express the DNA in neointimal cells, thereby

preventing or reducing the proliferation of neointimal cells. By "preventing or reducing neointimal cell proliferation" is meant that proliferation is decreased and can include sufficiently decreased as to constitute inhibition of proliferation. Any decrease can be measured by the methods taught herein. This method can be used to treat any condition in which injury to a blood vessel causes neointimal formation, such as primary atherosclerosis.

Thus, the present invention also includes a method of treating primary atherosclerosis in a blood vessel of a subject comprising preventing or reducing neointimal cell proliferation comprising administering to the blood vessel a replication-deficient recombinant adenovirus which functionally encodes a DNA which can decrease the proliferation of neointimal cells, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect and express the DNA in neointimal cells, thereby preventing or reducing the proliferation of neointimal cells and treating primary atherosclerosis. In particular, treatment of an existing atherosclerotic lesion with an adenovirus encoding the herpes simplex virus thymidine kinase gene along with ganciclovir treatment, as described herein, can be done to reduce the size of the atherosclerotic lesion. Any size reduction can be of benefit since it would allow for increased blood flow distal to the site of the lesion, and since such methods would provide an alternative to balloon angioplasty in reducing the size of primary atherosclerotic lesions.

Also provided by this invention is a method of reducing neointimal cell proliferation in an injured blood vessel of a subject comprising administering to the blood vessel (1) a replication-deficient recombinant adenovirus which functionally encodes herpes simplex virus thymidine kinase, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect neointimal cells, and (2) an effective amount of ganciclovir, thereby reducing the proliferation of neointimal cells expressing the herpes simplex virus thymidine kinase and in neighboring, nonexpressing neointimal cells. By an "effective amount" of ganciclovir is meant an amount sufficient to reduce or inhibit proliferation of neointimal cells, particularly in

conjunction with herpes simplex virus thymidine kinase expression. Any reduction in proliferation of neointimal cells can be useful. Such effective amounts can readily be determined following both the *in vitro* and *in vivo* examples provided herein. As described above, the administration of the adenovirus can be performed prior to injury, at the time of injury, or after injury to the blood vessel. A preferable administration period is from about 1 week prior to injury to about 4 weeks after injury. A more preferable administration period is from about the time of injury to about 20 days or from about the time of injury to about 15 days after the blood vessel is injured, and more preferably, from about the time of injury to about 7 days following injury. For practical considerations, it can be preferable to administer the adenovirus at the time of injury. Administration is preferably performed during the time period in which cells within the artery wall are proliferating.

Administration of the ganciclovir can be done prior to, concurrently with or after administration of the adenovirus. Additional doses of ganciclovir are preferably also administered. Administration of ganciclovir is preferably performed within from about 2 days prior to administration of the adenovirus to about 30 days after administration of the adenovirus, and more preferably within about 0 to about 15 days from administration of the adenovirus. Most preferably, several doses are adminstered, following administration of the adenovirus, in a series of doses over the given time periods. Most preferably, a dose of ganciclovir is administered once or twice daily for the duration of the administration of ganciclovir. The duration of administration of doses of ganciclovir can be from about one day until the time that proliferation is reduced or inhibited the desired amount, regardless of when treatment is started. Typically, administration will continue for from about one to about 30 days, and more preferably, from about one to about 15 days. Ganciclovir can be administered prior to adminstration of the adenovirus to elevate blood levels of ganciclovir prior to expression of the thymidine kinase. Such administration can be followed for analogous treatments, such as infection with cytosine deaminase and administration of 5-fluorocytosine.

A dose of ganciclovir or the like will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the disease that is being treated, the particular compound used, and its mode of administration. Thus, it is not possible to specify an exact amount. However, an appropriate amount may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. Generally, for ganciclovir the dosage will approximate that which is typical for the administration of ganciclovir for its approved indication in humans, and will preferably be in the range of from about 5 mg/kg/day to about 80 mg/kg/day, and more preferably, from about 10 to about 60 mg/kg/day, and more preferably, from about 20 to about 40 mg/kg/day.

The adenovirus and /or prodrug compositions can include, as noted above, an effective amount of the selected compound in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected compound without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

The initial amount of adenovirus necessary to deliver a therapeutic or prophylactic amount of DNA to human neointimal cells can be deduced from the rat data set forth herein and from similar experiments utilizing adenovirus for gene therapy for other pathologies. The dose can then be optimized using standard techniques.

The invention also provides a method of screening DNA for the ability to inhibit proliferation of or to have cytotoxic effects on neointimal cells comprising administering to an injured blood vessel in a subject at the site of injury a replication-deficient adenovirus which functionally encodes the DNA, for a time sufficient for the adenovirus to selectively infect neointimal cells; and detecting inhibition of proliferation of or toxicity to the neointimal cells, such inhibition or toxicity indicating a DNA having

the ability to inhibit proliferation of or to have cytotoxic effects on neointimal cells. Inhibition of proliferation of or toxicity to neointimal cells can be detected by any of several known methods, such as described herein. Naturally, such screening would preferably be performed in a non-human animal. In particular, evaluation of the ratio of neointimal to medial areas of histological sections obtained from treated arteries, as described herein, provides a useful standard measure for effectiveness of any treatment.

While adenovirus are utilized herein, the invention also contemplates the use of other viral vectors, so long as these vectors can selectively infect neointimal cells and selectively express the DNA encoded by the viral vector. Such vectors can be screened for utility using the methods taught herein.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

Statement Concerning Utility

The present method, which provides an effective method for selectively and efficiently expressing DNA in neointimal cells, has many uses both *ex vivo* and *in vivo* which are apparent and some of which are exemplified as follows. The method allows the selective targeting of dividing cells, and particularly neointimal cells, for the expression of exogenous nucleic acids. Thus proliferating cells can be targeted, for example, for detection purposes, for screening uses, for therapeutic purposes or for selective cytotoxicity. For example, proliferating cells in an injured artery or proliferating cells in the early stages of tumor formation can be detected by selectively expressing in these cells a detectable gene product. Furthermore, this method can be used in *ex vivo* methods for screening compounds that affect expression of a transfected gene in proliferating cells or that affect the action of the transfected gene product in proliferating cells. Additionally, neointimal cells can be targeted for selective antiproliferative effects in subjects having vascular injury, such as resulting from balloon

angioplasty, distal peripheral arterial reconstruction, atherectomy, stent implantation and laser angioplasty. Such targeting can be useful to delay, decrease or inhibit restenosis. In particular, the herpes simplex virus thymidine kinase gene can be selectively introduced into neointimal cells and then ganciclovir administered to selectively inhibit DNA synthesis, and thus cell proliferation, in neointimal cells.

EXAMPLES

Adenovirus Vectors. The replication deficient recombinant adenovirus (Ad.RSVβgal) has previously been described (28,33). Briefly, the recombinant virus encodes for the histochemical marker gene β-galactosidase (β-gal). The gene, derived from *E. coli*, has been modified by a eukaryotic nuclear translocation signal and placed under the control of the Rous Sarcoma Virus (RSV) long terminal repeat. Expression of the β-gal gene product results in a nuclear dominant blue staining pattern when cells are exposed to the chromogen 5-bromo-4-chloro-3 indoyl β-D-galactopyranoside (X-gal). A similar adenovirus containing the human cystic fibrosis transmembrane conductance regulator cDNA (AdCFTR) (29), whose recombinant gene product does not react with the X-gal chromogen, was used as a control. Viral stocks (1.5×10^{10} pfu/ml) were prepared by passaging recombinant adenovirus in 293 cells. (31, 39)

The AdMLP.HSTK vector, functionally encoding the herpes simplex virus thymidine kinase gene (HS-tk), was constructed by performing homologous recombination in 293 cells. The 1.8 kb cDNA encoding HS-tk was ligated into an adenovirus shuttle plasmid. This shuttle plasmid based on pBluescript II SK (Stratagene, LaJolla, California) contained a multiple cloning site (mcs) flanked on the 5' end by the left inverted terminal repeat (ITR) of adenovirus, the E1A enhancer, along with the Major Late Promoter (MLP) and tripartite leader of adenovirus type 2. On the 3' end of the mcs there was an approximate 3 kb region homologous to adenovirus type 5 (Ad 5). A linearized fragment of this recombinant plasmid was cotransfected into 293 cells along with *ClaI* cut adenovirus DNA from the E3 deletion mutant Ad-dl327. Single plaques were amplified in 293 cells. Recombinant virus was screened using

polymerase chain reaction amplification for the presence of HS-tk and the absence of the E1 region to demonstrate successful homologous recombination. A single PCR positive plaque, AdMLP.HSTK, was amplified in 293 cells and subsequently purified on cesium gradients. Viral stocks were titered on 293 cells by standard methods. Cells infected with AdMLP.HSTK showed the expected size transcript and protein on Northern and Western blots.

Cell Culture. Vascular SMCs were isolated from a rat thoracic aorta by enzymatic digestion as previously described (40). Cells were maintained in M199 medium (Biofluids, Gaithersburg, Maryland) containing 10% FBS (Biofluids, Gaithersburg, Maryland) and 1X penicillin/streptomycin in humidified air containing 5% CO_2 at 37°C. Cells were routinely passaged just before reaching confluence by brief exposure to Puck's saline solutions containing 0.25 mg/ml trypsin (Biofluids, Gaithersburg, Maryland) and 0.5 mM EDTA. At passage 3, cells were placed on 8 well microchamber slides and stained for alpha smooth muscle actin (Sigma Immunochemicals, Co., St. Louis, Missouri) to confirm their identity.

***In Vitro* Gene Transfer**. Early passage (P4) SMCs were plated in duplicate on T-25 culture flasks and grown to 30% confluence. They were then placed in media containing 0.5% serum and allowed to become quiescent over 36 hrs. The cells were then washed twice in phosphate buffered saline (PBS) and then either (a) incubated for 5, 15, 30, 60 or 120 minutes in 2 ml of media containing 2.5 X 10^7 pfu/ml Ad.RSVβgal (which was equivalent to approximately 100 pfu per cell) or (2) incubated for 120 minutes with 0, 1, 10, or 100 pfu/cell of AdMLP.HSTK . After the given interval, the cells were washed three to five times in PBS and incubated in 5 ml of media containing 0.5% serum for 24 hrs.

For cells infected with Ad.RSVβgal, prior to staining in X-gal solution, cells were washed and fixed for 5 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS pH 7.4. The percent of transfected cells was calculated by counting 500 cells in

each of two duplicate flasks and noting the number of cells with nuclear dominant blue staining. Percentages represent an average for 2 flasks at each time point.

For cells infected with AdMLP.HSTK, after the 24 hour period in 0.5% serum medium, the cells were exposed (at this point marked as Day 0) to medium containing 10% serum and 0, 1, or 10 μg/ml ganciclovir.

Animals. All animals were studied under protocols approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute and in accordance with the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services publication No. [NIH] 86-23, revised 1985).

Adult Sprague-Dawley rats weighing 350-450 g (Taconic farms, Germantown, New York) were used for these experiments. All procedures were performed under general anesthesia and using sterile technique. General anesthesia was administered using ketamine 150 mg/kg and xylazine 15 mg/kg IM and supplemental ketamine/xylazine IP as necessary. Beef lung heparin (Upjohn, Kalamazoo, Michigan) was routinely given IV (100 u/kg) prior to cross clamping the vessel for virus incubation. All viral incubations consisted of instilling a solution containing 0.5-1.0 X 10^9 pfu of adenovirus in a total volume of 100 μl for 45 minutes. Medium M199 (Biofluids, Gaithersburg, Maryland) was used to dilute stock viral solution to the appropriate concentration. Upon completion of each procedure, animals were allowed to recover with free access to food and water.

***In Vivo* Gene Transfer Into Arterial Segments**. Adenoviral-mediated gene transfer was first evaluated in uninjured arterial segments (n=4). In each rat the left carotid artery was exposed, proximal and distal control obtained, and an arteriotomy was made in the external carotid. A solution containing either Ad.RSVβgal or AdCFTR was instilled through the external carotid using a 24 g catheter (Criticon Inc., Tampa, Florida). The solution was then evacuated, the external carotid was ligated, and the

incision was closed. All rats in this group were sacrificed at 3 days after infection and the carotid was subsequently harvested, fixed and stained as described below.

To assess gene transfer to areas of vascular injury, i.e., including areas of neointimal cell formation, the left carotid artery of rats was exposed and injured as previously described (41, 42). Briefly, the common carotid and its external branch were exposed and encircled using 4-0 silk ties. An arteriotomy was then made in the external carotid artery and a 2F Fogarty embolectomy catheter was passed into the common carotid, filled with 0.2 cc of air and passed back and forth 3 times. The carotid artery of one group of rats (n=7) was exposed to virus immediately after injury. Adenoviral solution was delivered into the common carotid lumen via the external carotid artery. After the 45 minute incubation, the external carotid was ligated, the carotid reperfused, and the animal was allowed to recover as previously described.

In three other groups of animals, the arteries were exposed to virus either 3 (n=9), 7 (n=9) or 12 (n=4) days following injury. These animals were re-anesthetized following the prescribed time interval after injury. The common carotid was exposed through the previous incision and a portion of the common carotid measuring approximately 1.3 cm was isolated between 1mm microvascular clamps. An arteriotomy was made in the common carotid using a 24 g catheter and the vessel was irrigated with 50-100 µl of normal saline. Adenoviral solution containing Ad.RSVβgal, **AdMLP.HSTK,** or control virus was delivered directly into the carotid lumen. At the conclusion of the viral incubation, the catheter was removed and the arteriotomy was closed using 3 interrupted 10-0 ethilon stitches (Ethicon Inc., Somerville, New Jersey). The incision was closed and the animal was allowed to recover as described above.

Ganciclovir Treatment. Cells to be exposed to ganciclovir were incubated in medium containing 0, 1, 10, or 100 µg/ml, as indicated. For standard experiments, unless indicated otherwise, 10 µg/ml was used. Animals to be treated with ganciclovir were administered, unless otherwise indicated, 30 mg/kg ganciclovir twice a day intraperitoneally.

Evaluation of *In Vivo* Gene Transfer. In 30 animals, gene transfer was routinely assessed three days after exposure to adenovirus. One group of animals (n=3) which underwent infection 7 days after injury, were sacrificed two weeks after infection to determine persistence of gene expression. Animals were sacrificed by overdosing with pentobarbital.

The carotid artery was harvested and cut longitudinally to expose the lumen. It was then washed in 2 ml of PBS and fixed for 5 min in a solution containing 2% formaldehyde and 0.2% glutaraldehyde in PBS. The artery was washed in PBS several times to remove any excess fixative and, for AdRSV.βgal infected animals, placed into X-gal solution (5mM $K_4Fe(CN)_6$, 5mM $K_3Fe(CN)_6$, 1mM $MgCl_2$, and 1 mg/ml X-gal in PBS) for 4 hrs. For histologic examination, carotid arteries were cut into 2 mm segments, embedded in paraffin, cut into 5 μm sections, and counterstained with nuclear fast red. In order to estimate gene transfer efficiency in histologic sections, neointimal cells were counted in at least 3 high power (400X) fields from 3 or more histologic sections of each test artery and the percentage of nuclear dominant blue cells was calculated. At least 300 cells were counted in each section.

In two separate sections containing neointima, immunohistochemical staining with an antibody to alpha smooth muscle actin was performed. This analysis demonstrated cytoplasmic staining of most neointimal cells and medial smooth muscle cells, a finding in agreement with previous studies that identified neointimal cells as being primarily of SMC origin (41). In three of the animals that were infected three days after injury and sacrificed 3 days later, the brain, heart, and liver were also harvested to determine if gene expression in distal organs was detectable. These specimens were washed in PBS, fixed in 2% formaldehyde, 0.2% glutaraldehyde for 20 minutes and stained in X-gal solution for 6 hrs. Histologic sections were counterstained using nuclear fast red.

RESULTS

***In Vitro* Gene Transfer**. We first sought to assess the efficiency of adenoviral mediated gene transfer in cultured primary rat aortic SMCs. β-gal expression was detected in approximately 25% of the SMCs after a 5 min exposure to adenovirus. With increasing time of incubation, a higher percentage of cells stained positive (Fig. 1). With a 1 hr exposure, close to 70% of cells appeared positive. As expected, the predominant staining was intranuclear, since the β-gal gene was modified by the addition of a nuclear localizing sequence. A nuclear dominant blue stain was not seen in mock infected cells. As noted by others (21, 24), however, we noted occasional faint blue cytoplasmic staining in both infected and control cells of vascular origin. Although these results were obtained on quiescent cells, qualitatively similar results were obtained in cells maintained in normal growth media (data not shown).

Growth of in vitro thymidine kinase-transfected cells. As described above, the growth was determined of vascular SMCs infected with either (A) AdMLP.HSTK (100 pfu/cell) (Fig. 2) or (B) Ad RSV.βgal (100 pfu/cell) (Fig. 3), and then treated with increasing concentrations of ganciclovir (gcv), as indicated. Values are plotted as mean ± standard error, with each point performed in triplicate.

The growth of vascular SMCs infected at various multiplicities of infection with AdMLP.HSTK, then treated with 10 μg/ml ganciclovir was determined, as described above. Data is provided in Fig. 4. Values are plotted as mean ± standard error, with each point performed in triplicate.

The above *in vitro* experiments, some of the data of which is provided in Figs 2, 3, and 4, showed that treatment did inhibit SMC proliferation, and that inhibition was dependent on both the concentration of ganciclovir used and the multiplicity of infection (m.o.i.) of AdMLP.HSTK. Cells infected with 100 m.o.i. AdMLP.HSTK and exposed to medium containing no ganciclovir showed a normal proliferation curve. However, cells infected similarly but exposed to 1 μg/ml of ganciclovir showed a reduction in cell number of approximately 70% at two weeks, while those cells exposed to ganciclovir at a concentration of 10 μg/ml showed a complete inhibition in proliferation. This same concentration range of ganciclovir produced only a modest effect on cell growth when SMCs were infected with 100 m.o.i. of the control adenovirus, AdRSV.ßGAL. The antiproliferative effect of AdMLP.HSTK was also dependent on the multiplicity of infection (m.o.i.) used. In cells exposed to ganciclovir at a concentration of 10 μg/ml, growth inhibition increased as the m.o.i. increased from 0 to 100 pfu/cell.

Bystander Effect. To assess whether primary rat SMC were susceptible to a "bystander" effect, wherein cells expressing the HS-tk gene exert an antiproliferative effect on neighboring cells that do not express HS-tk, cells were infected (100 m.o.i.) with either AdMLP.HSTK or AdRSV.βgal and then mixed and replated (day 0) in varying proportions. Since we had previously shown *in vivo* that between 10%-75% of neointimal cells were successfully transduced by adenoviral infection, similar ratios of HS-tk infected and noninfected cells were prepared *in vitro*. The following day (day 1), 10 μg/ml of ganciclovir was added to the media. At the end of seven days, the fold-increase in cell number from day 1 was determined. AdRSV.βgal infected cells which were not mixed with AdMLP.HSTK infected cells demonstrated an approximately 20-fold increase in number. Cells infected with AdMLP.HSTK alone showed an almost

complete inhibition of cell growth over the seven day period. Though cells infected with AdMLP.HSTK alone had the greatest growth inhibition, a 50:50 mix of AdMLP.HSTK infected and AdRSV.βgal infected cells were inhibited almost to the same degree suggesting an effect on untransfected cells.

All of these *in vitro* data indicate that even though the adenovirus genome does not integrate into the host cell genome and even in the absence of 100% Hstk gene transfer, adenovirus-mediated gene transfer of HS-tk can allow for inhibition of SMC proliferation and this negative selective pressure can be maintained for at least 2 weeks.

***In Vivo* Gene Transfer Into Uninjured Arterial Segments**. By extrapolating from the *in vitro* time frame data, the vessel wall was next exposed to adenoviral solution for 45 min. With this incubation period, transfection efficiency appeared to be within the plateau region of the *in vitro* data curve (Fig. 1). β-gal gene expression in uninjured arterial segments (n=2) could be detected on gross examination when assessed three days after infection. Histological examination showed β-gal staining of scattered endothelial cells throughout the region incubated with adenovirus. Staining was also occasionally noted in cells of the adventitia. No medial SMCs stained for β-galactosidase activity. This suggests that the endothelium or the internal elastic lamina may form a barrier to adenoviral infection of medial cells. No β-gal staining was observed in arteries infected with the AdCFTR control virus (n=2).

***In Vivo* Gene Transfer Into Injured Arterial Segments**. In order to assess the efficiency of gene transfer in injured arterial segments, and to determine whether neointimal cells have different susceptibility to gene transfer than medial SMCs, the rat carotid injury model was used. In this model, balloon abrasion removes the endothelial layer and disrupts one or more layers of internal elastic lamina (41). The injury activates medial SMCs, which begin to migrate and proliferate to form a neointima between 3 and 5 days after injury. A well formed neointima is routinely

present at 12 days after injury. For this reason we assessed gene transfer immediately, 3 days, 7 days and 12 days after balloon injury.

AdRSV.βgal. In carotid segments where virus was instilled immediately after injury, only 3 of 5 segments showed gross evidence of β-gal expression. The staining was, in general, limited to less than 5% of the vessel surface. Histological examination confirmed that no endothelial layer existed. Despite this, only occasional medial SMCs positively stained for β-gal (data not shown). Similar results were obtained when infection was delayed to 3 days after injury. In this group only 4 of 7 carotid vessels incubated with Ad.RSVβgal showed gross evidence of β-gal staining. This was again limited to less than 5% of the vessel surface. Histology revealed only occasional neointimal or medial SMC staining. Control segments treated with AdCFTR either immediately (n=2) or 3 days after injury (n=2) showed no staining for β-gal activity.

In contrast, markedly increased β-gal activity was observed in segments in which infection was delayed until 7 days after injury. At this time, we found that neointima covered most, but not all of the arterial surface. Intense staining, indicative of gene transfer and expression, was evident on gross examination in each of the arterial segments obtained from these animals exposed to β-gal containing adenovirus. Histological examination showed staining limited almost exclusively to the neointima. We examined multiple histologic sections containing neointima from each of the animals infected 7 days after injury. The efficiency of gene transfer was noted to vary from approximately 20% to over 75% of neointimal cells. In the majority of sections, over 50% of neointimal cells expressed β-gal. Surprisingly, neointimal cells were selectively targeted, as we observed that very few cells located deep to the first layer of internal elastic lamina stained positive for β-gal, whether or not overlying neointima was present. Arterial segments infected 7 days after injury with control AdCFTR virus (n=3) showed no visible staining on gross or microscopic examination.

In the final group in this series of experiments, we exposed arteries to Ad.RSVβgal at 12 days after injury and harvested the vessels 3 days later (n=2). At this

point, the neointima is thicker and covers most of the arterial surface. Each of these segments showed uniform staining of the luminal surface. Histology again showed efficient gene transfer that was selective for neointimal cells. In general, the cells located in the more superficial portions of the neointima appeared to have a higher efficiency of gene transfer. Carotid segments in which animals were infected 12 days after injury with the control adenovirus AdCFTR showed no evidence of staining (n=2).

In the three animals in which distal organs (brain, liver, and heart) were harvested in order to evaluate gene expression at distal sites, no β-gal staining could be visualized in any area by gross or histologic evaluation.

Persistence of Gene Expression. In a separate group consisting of three animals, we made a preliminary assessment of the persistence of gene expression. Rats were infected seven days after injury and their carotid arteries were harvested two weeks later. Analysis of β-gal expression in these segments showed persistence of β-gal activity in each of the three animals. However, relative to segments harvested 3 days after infection, expression was qualitatively diminished.

AdMLP.HSTK. To assess whether local infection of AdMLP.HSTK along with concomitant ganciclovir administration could inhibit vascular neointimal cell accumulation *in vivo*, rats underwent balloon injury of the left carotid artery as described by Clowes et. al. (41). Seven days after injury, 100 µl of a solution containing 3×10^8 or 1×10^9 pfu of adenovirus was instilled into the vessel as described above. After 50 min., the viral solution was withdrawn, the arteriotomy was closed, and the rats were allowed to recover. The following day treatment was initiated with either ganciclovir (30 mg/kg intraperitoneally twice daily) or H_2O as a control. Treatment was continued for 14 days at which time the animals were sacrificed and the carotid arteries harvested for histologic assessment of the amount of injury-induced neointimal development.

Six groups consisting of a total of 73 Sprague-Dawley rats were analyzed. Four control groups were treated with: (1) vehicle only, no ganciclovir; (2)

AdRSV.ßGAL (1 x 10^9 pfu), no ganciclovir; (3) AdMLP.HSTK (1 x 10^9 pfu), no ganciclovir; and (4) AdRSV.ßGAL (1 x 10^9 pfu) with ganciclovir. The two treatment groups consisted of AdMLP.HSTK (3 x 10^8 pfu) with ganciclovir or AdMLP.HSTK (1 x 10^9 pfu) with ganciclovir.

Examination of histological sections from animals in the four control groups all showed appreciable neointimal formation when compared to uninjured arteries. In contrast, the neointimal mass appeared qualitatively reduced in those animals infected with AdMLP.HSTK and treated with ganciclovir, thus indicating effectiveness against restenosis.

To quantify the apparent reduction in neointimal area seen in the two treatment groups, the ratio of neointimal/medial area in injured arteries was determined using digital planimetry as known in the art. Neointimal and medial areas were determined at 21 days after injury by digital planimetry using the section with the greatest neointimal area. Sections were read by a single observer in a blinded fashion. Six groups of animals described above were studied, with the number of animals studied in each group shown in Fig. 5, which depicts the average ratio ± standard error.

The mean neointimal/medial ratios in the four control groups were statistically indistinguishable. In contrast, administration of AdMLP.HSTK with a fixed dose of ganciclovir significantly reduced the mean neointimal/medial ratio (ANOVA $p=0.027$). The observed reduction in neointimal/medial ratio was entirely dependent on reduction of the neointimal area, as the medial area did not differ significantly among the six groups (ANOVA $p = 0.38$).

The magnitude of neointimal reduction in the treated groups was dependent on the amount of AdMLP.HSTK used (Fig. 5). Compared to the four control groups, animals infected with the lower dose of 3 x 10^8 pfu AdMLP.HSTK and treated with systemic ganciclovir had an approximately 20% reduction of the neointimal/medial ratio, a reduction which approached statistical significance ($p = 0.058$). At the higher dose of

1×10^9 pfu AdMLP.HSTK, the reduction of neointimal/medial ratio was 46% ($p < 0.01$). Linear regression analysis of percent neointimal reduction versus adenovirus dose was performed using 3 groups (vehicle, low dose AdMLP.HSTK, and high dose AdMLP.HSTK). This analysis revealed a slope significantly different from zero ($p=0.004$) indicative of a dose response relationship. This dose-response relationship suggests that higher doses of AdMLP.HSTK may produce additional benefits.

In the data presented above for β-gal expression, in uninjured vessels, gene transfer and expression were confined predominantly to endothelial cells and adventitial cells. Only rare medial SMCs expressed the β-gal gene product. Adventitial staining was often found around the vasa vasorum, which suggests the virus may have entered the vasa vasorum and thereby gained access to, and infected, the surrounding cells. Another possibility is that minute quantities of viral solution could have been inadvertently spilled on the adventitial surface during the procedure. The efficiency of staining in uninjured vessels appeared to be considerably less impressive than was previously seen in sheep carotid segments (36). Furthermore, when the presence of viral DNA was assessed in distal organs of animals infected as herein described, by extracting DNA from the liver, spleen, myocardium, gonadal tissue, and infected carotid segment 48 hours after infection and performing standard polymerase chain reaction (PCR) on the extracted DNA, the results showed no adenoviral DNA detected in any organ other than the infected carotid artery segment.

In injured vessels, we noted a difference in gene transfer efficiencies depending upon when after injury the artery was exposed to virus. At early time points after injury (3 days or less), when endothelial cells were absent and there were few neointimal cells, efficiency of gene transfer was relatively low and expression was confined to scattered medial and neointimal cells. Exposing vessels to adenovirus 7 days or 12 days after injury, during which time significant neointima had formed, we found a markedly increased efficiency of β-gal gene transfer and expression. Surprisingly, however, most of the cells expressing the β-gal gene product were located on the

luminal surface of the internal elastic lamina; i.e., the transfected cells were almost exclusively neointimal cells.

The mechanism responsible for this difference in susceptibility between neointimal and medial SMCs to adenoviral-mediated gene transfer and expression is unclear. While not limiting this invention, the most probable explanation is that a physical barrier, most likely the internal elastic lamina, prevents diffusion of virus into medial layers. Thus, neointimal cells, which reside on the luminal side of the internal elastic lamina are able to be infected, while medial SMCs, which reside deep to the internal elastic lamina, are not. Alternatively, the process of injury induced neointimal proliferation may affect the expression of the as yet uncharacterized adenoviral cell surface receptor. Increased expression of the target viral receptor by proliferating neointimal cells could explain their efficient and selective uptake of adenovirus. Of note, however, in our *in vitro* experiments, we found no appreciable difference in adenoviral transduction between quiescent and proliferating SMCs.

In this study, we routinely evaluated gene expression 3 days after infection. In 3 segments, however, we assessed β-gal staining 2 weeks after infection. These segments showed continued, but diminished, expression of the marker gene. Previous studies in endothelial cells *in vitro* have shown that gene expression peaks around 7 days after infection and persists for at least 14 days (43). The same is true in endothelial cells *in vivo* (36). Long-term gene expression would not be expected from adenoviral gene transfer, since the virus does not stably integrate into the genome of the host cell. Such a time frame of gene expression, while a potential drawback in the treatment of inherited genetic disease, can be an advantage for the treatment of a temporally discrete event such as restenosis. Any treatment strategy that uses recombinant adenovirus must address the question of safety. The general lack of stable integration of adenovirus, as opposed to retroviruses, is a significant advantage in that issues of insertional mutagenesis are of less concern (37). The safety of adenovirus in humans has been tested in vaccine trials in the past (44-47) and presently in ongoing clinical trials in the treatment of cystic fibrosis (48). Although we made no concerted

effort to evaluate distal organs in all animals exposed to Ad.RSVβgal, we could not detect β-gal expression in the brain, liver, or heart of each of 3 animals in which these organs were harvested. This demonstrates that adenoviral gene transfer can in large part be limited to discrete vascular segments without subjecting distal organs to infection.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(I) TELEX: None

(ii) TITLE OF INVENTION: Efficient and Selective Adenoviral-Mediated Gene Transfer into Vascular Neointima

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: None yet assigned
(B) FILING DATE: 13-OCT-1994

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/136,113
(B) FILING DATE: 13-OCT-1993

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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What is claimed is:

1. A method of selectively expressing DNA in neointimal cells in an injured blood vessel of a subject comprising administering a replication-deficient recombinant adenovirus, which functionally encodes the DNA, to the blood vessel at the site of injury, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect neointimal cells and thereby selectively express the DNA in neointimal cells.

2. The method of Claim 1, wherein the administration step is performed at the time the blood vessel is injured.

3. The method of Claim 1, wherein the administration step is performed from about 0 to about 12 days after the blood vessel is injured.

4. The method of Claim 1, wherein the administration step is performed from about 0 to about 7 days after the blood vessel is injured.

5. The method of Claim 1, wherein the time the adenovirus remains at the site of injury is from about 15 minutes to about 60 minutes.

6. The method of Claim 1, wherein the DNA encodes a protein.

7. The method of Claim 1, wherein the DNA encodes an antisense ribonucleic acid.

8. A method of treating restenosis in an injured blood vessel of a subject comprising administering to the blood vessel a replication-deficient recombinant adenovirus which functionally encodes a DNA which can decrease the proliferation of neointimal cells, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect and express the DNA in neointimal

cells, thereby decreasing or inhibiting the proliferation of neointimal cells and treating restenosis.

9. The method of Claim 8, wherein the administration step is performed at least about 4 days after the blood vessel is injured.

10. The method of Claim 8, wherein the DNA encodes a protein.

11. The method of Claim 8, wherein the DNA encodes an antisense ribonucleic acid.

12. The method of Claim 10, wherein the protein is selected from the group consisting of herpes simplex thymidine kinase, cytosine deaminase, dominant negative ras gene product and nitric oxide synthase.

13. The method of Claim 11, wherein the antisense ribonucleic acid is derived from the group consisting of c-myc, c-myb, CDC2 and PCNA.

14. The method of Claim 8, wherein the DNA is cytotoxic to the neointimal cells.

15. A method of preventing or reducing neointimal cell proliferation in an injured blood vessel of a subject comprising administering to the blood vessel a replication-deficient recombinant adenovirus which functionally encodes a DNA which can decrease the proliferation of neointimal cells, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect and express the DNA in neointimal cells, thereby preventing the proliferation of neointimal cells.

16. A method of treating primary atherosclerosis in a blood vessel of a subject comprising preventing or reducing neointimal cell proliferation by the method of Claim 4.

17. A method of screening DNA for the ability to inhibit proliferation of or to have cytotoxic effects on neointimal cells comprising:

a. administering to an injured blood vessel in a subject at the site of injury a replication-deficient adenovirus which functionally encodes the DNA, for a time sufficient for the adenovirus to selectively infect neointimal cells; and

b. detecting inhibition of proliferation of or toxicity to the neointimal cells, such inhibition or toxicity indicating a DNA having the ability to inhibit proliferation of or to have cytotoxic effects on neointimal cells.

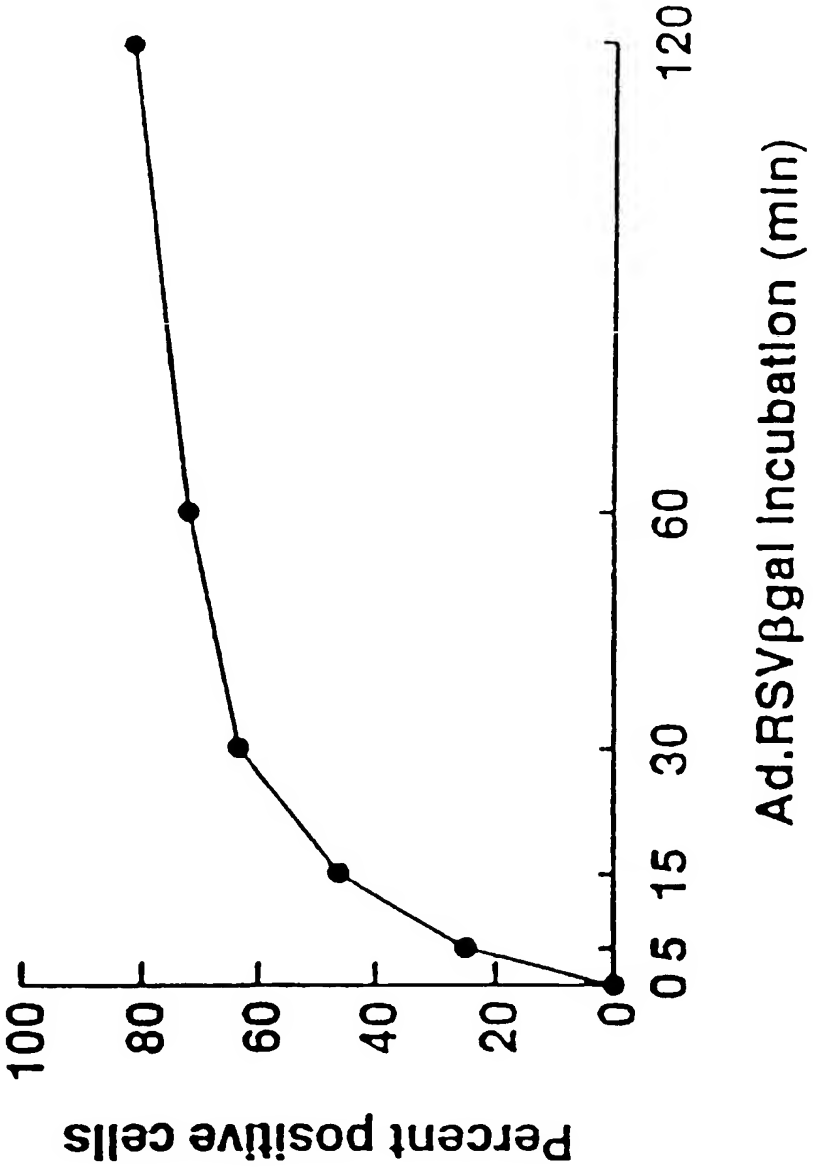
18. A method of reducing neointimal cell proliferation in an injured blood vessel of a subject comprising administering to the blood vessel (1) a replication-deficient recombinant adenovirus which functionally encodes herpes simplex virus thymidine kinase, such that the adenovirus remains at the site of injury for a time sufficient for the adenovirus to selectively infect neointimal cells, and (2) an effective amount of ganciclovir, thereby reducing the proliferation of neointimal cells.

19. The method of Claim 18, wherein the replication-deficient recombinant adenovirus is administered at any point within about 1 day prior to injury to about 15 days after injury to the blood vessel.

20. The method of Claim 19, wherein the replication-deficient recombinant adenovirus is administered at any point within about 0 to about 7 days after injury to the blood vessel.

21. The method of Claim 18, wherein the ganciclovir is administered in a series of individual doses.

FIG. 1


Percent positive cells
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40
20
0
0.5
15
30
60
120
Ad.RSVβgal Incubation (mIn)

FIG. 2

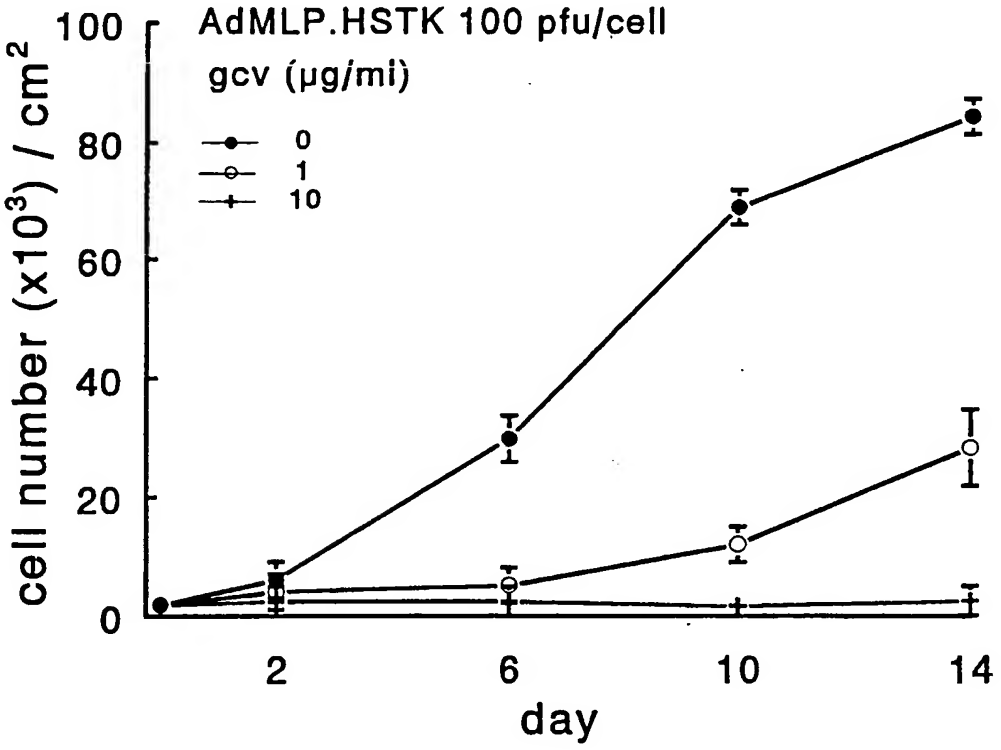
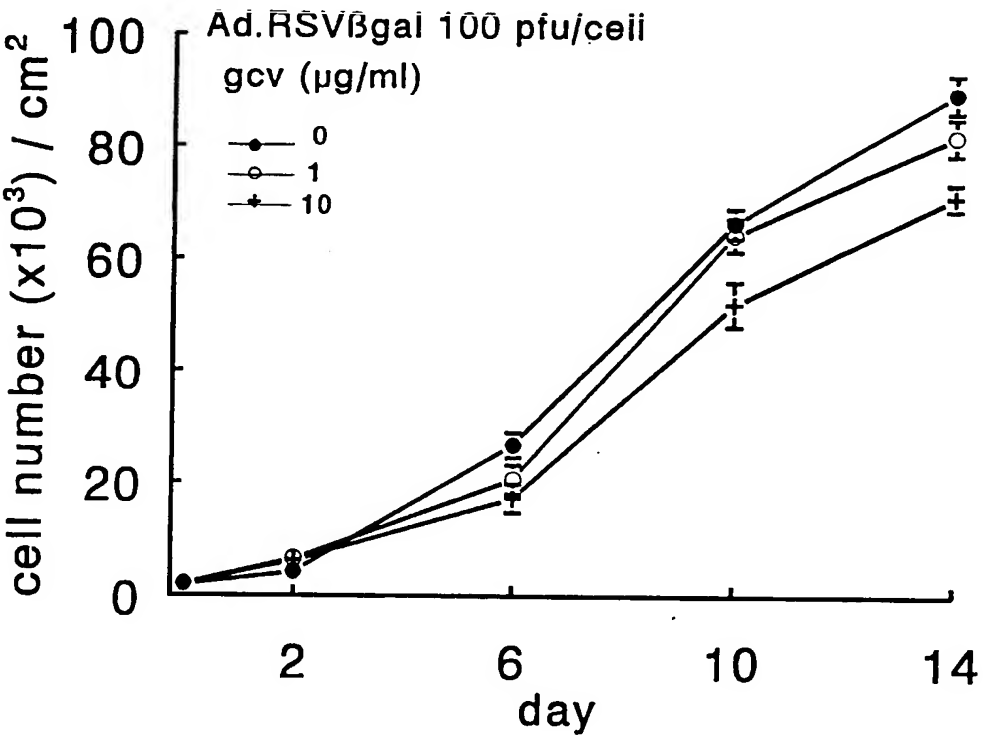

AdMLP.HSTK 100 pfu/cell
gcv (μg/ml)
0
1
10
cell number (x10³) / cm²
100
80
60
40
20
0
2
6
10
14
day

FIG. 3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gcv (µg/ml)
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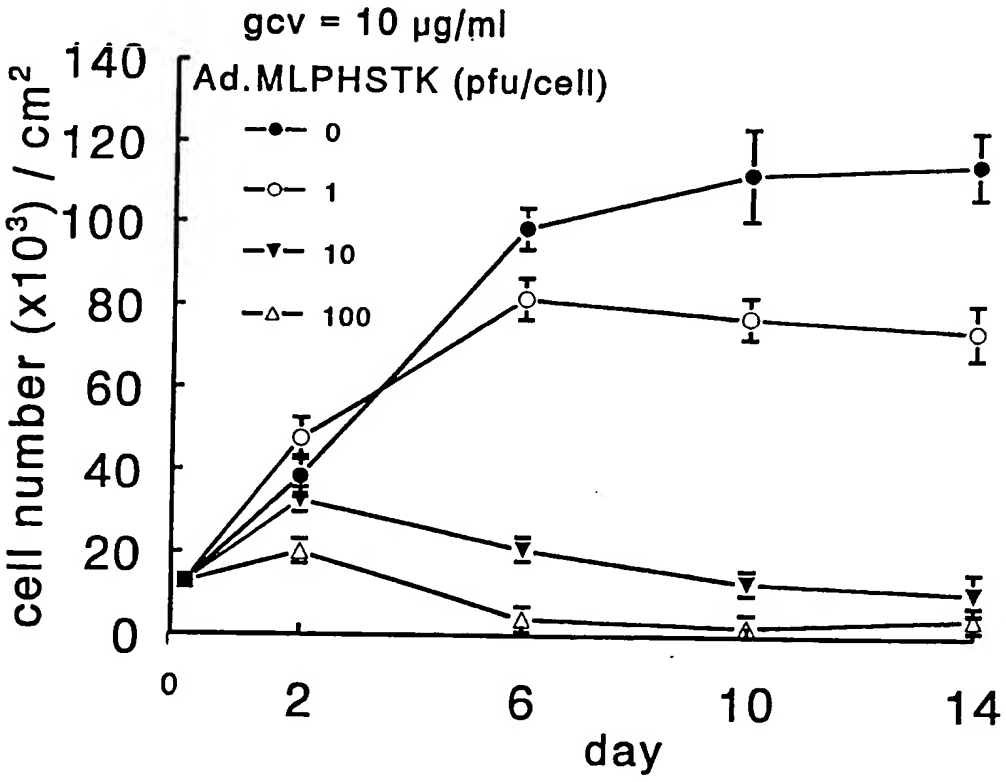
100
80
60
40
20
0
2
6
10
14
day

FIG. 4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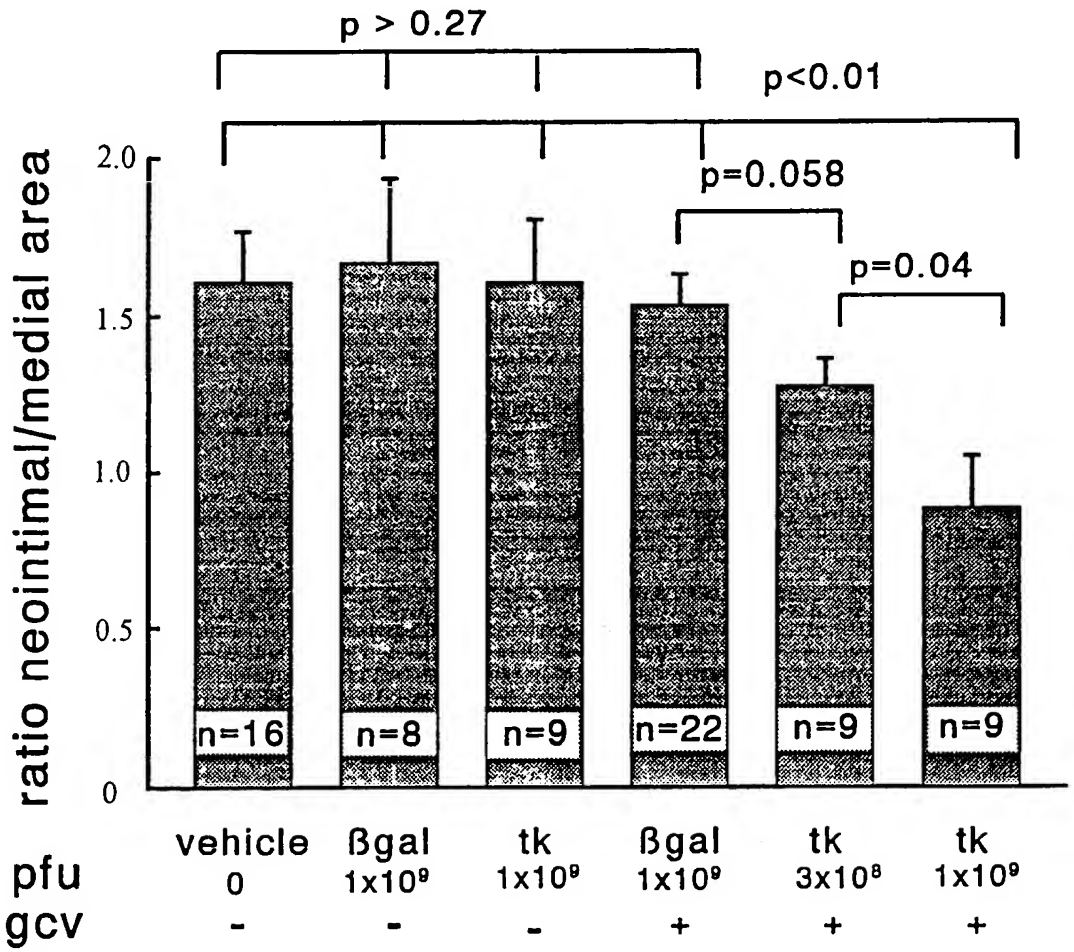
Ad.MLPHSTK (pfu/cell)
0
1
10
100
cell number (x10³) / cm²
140
120
100
80
60
40
20
0
0
2
6
10
14
day

FIG. 5


p > 0.27
p<0.01
p=0.058
p=0.04
ratio neointimal/medial area
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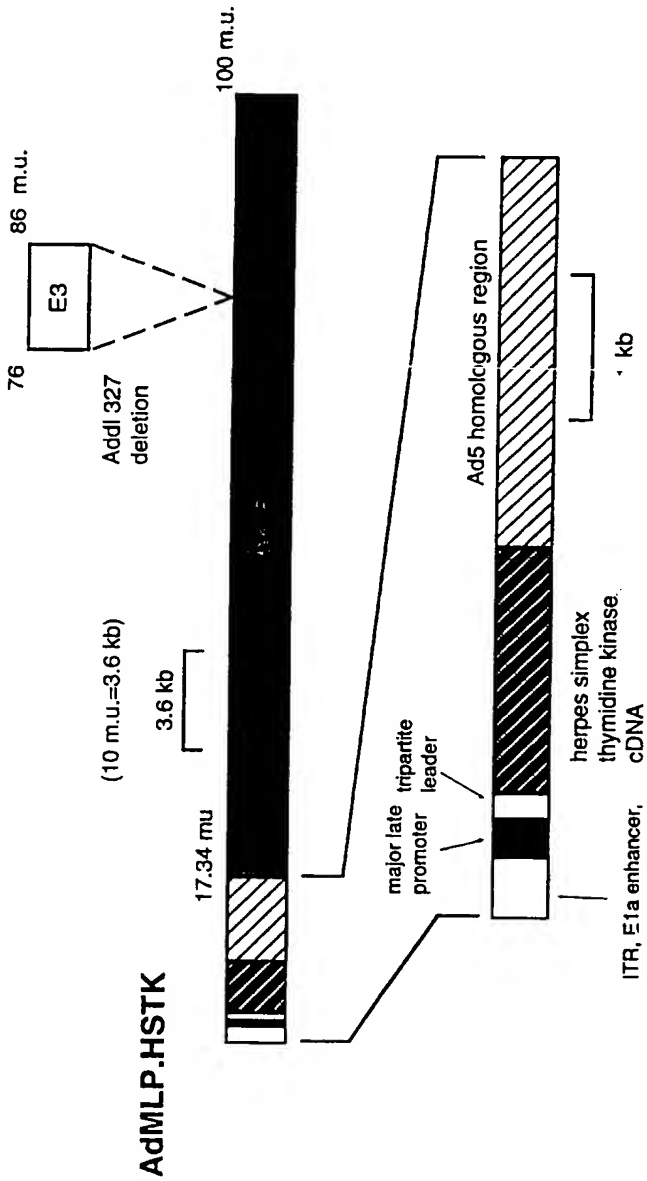
1.5
1.0
0.5
0
n=16
n=8
n=9
n=22
n=9
n=9
vehicle
ßgal
tk
ßgal
tk
tk
pfu
0
1x10⁹
1x10⁹
1x10⁹
3x10⁸
1x10⁹
gcv
-
-
-
+
+
+

FIG. 6


100 m.u.
86 m.u.
E3
76
AddI 327
deletion
(10 m.u.=3.6 kb)
3.6 kb
17.34 mu
AdMLP.HSTK
major late
promoter
tripartite
leader
Ad5 homologous region
kb
herpes simplex
thymidine kinase
cDNA
ITR, E1a enhancer,
encapsidation
signal

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 94/11676

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CIRCULATION, vol.88, no.4 P2, 4 October 1993 page I-80 GUZMAN, R.L. ET AL. 'Efficient and selective adenoviral-mediated gene transfer into vascular neointima' see Abstract 0419 ---	1-21
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.91, October 1994, WASHINGTON US pages 10732 - 10736 GUZMAN, R.J. ET AL. 'In vivo suppression of injury-induced vascular smooth muscle cell accumulation using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene' see the whole document --- -/--	1-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 1995	03.03.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Chambonnet, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 94/11676

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CIRCULATION, vol.90, no.4 P2, October 1994 page I-292 GUZMAN, R.J. ET AL. 'Inhibition of in vivo neointimal proliferation using adenoviral gene transfer of the Herpes Simplex Thymidine Kinase gene' see Abstract 1569	1-21
P,X	CIRCULATION, vol.88, no.6, December 1993 GUZMAN, R.J. ET AL. 'Efficient and selective adenovirus-mediated gene transfer into vascular neointima' see the whole document	1-21
P,X	CIRCULATION RESEARCH, vol.73, no.6, December 1993 pages 1202 - 1207 GUZMAN ,R.J. ET AL. 'Efficient gene transfer into myocardium by direct injection of adenovirus vectors' see the whole document	1
P,X	CIRCULATION RESEARCH, vol.73, no.5, November 1993 pages 797 - 807 LEE S.W. ET AL. 'In vivo adenoviral vector-mediated gene transfer into balloon-injured rat carotid arteries' see the whole document	1-21
P,X	WO,A,94 11506 (ARCH DEVELOPMENT CORPORATION) 26 May 1994	1,6,7
P,Y	see the whole document	8,10,11, 13,15-17
Y	EP,A,0 494 776 (UNIVERSITY OF EDINBURGH) 15 July 1992 see the whole document	1-21
T	WO,A,94 24297 (RHONE-POULENC RORER) 27 October 1994 see the whole document	8,10,11, 13,15-17
Y	JOURNAL OF VIROLOGY, vol.57, no.1, January 1986 pages 267 - 274 HAJ-AHMAD, Y. & GRAHAM, F.L. 'Development of a helper-independent human adenovirus vector and its use in the transfer of the Herpes Simplex Virus Thymidine kinase gene' see the whole document	1-21

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11676

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 8-16, 18-21 and, as far as they concern a method of treatment 1-7, are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Intern al Application No
PCT/US 94/11676

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9411506	26-05-94	AU-B-	5609394	08-06-94
EP-A-0494776	15-07-92	NONE		
WO-A-9424297	27-10-94	FR-A-	2704234	28-10-94
		AU-B-	6572194	08-11-94